Pestic. Sci. 1996, **46**, 247–253

Cholinergic Activity of Selected Methanesulfonate Insecticides. A Pharmacological Profile

Karen W. Smith,*[a] Christopher T. Virgona,[b] Jocelyn N. Pennefather[c] and George Holan[a]

[a] CSIRO, Division of Chemicals & Polymers, Bayview Ave, Clayton, Victoria, 3169, Australia
[b] CSIRO–CERIT, 2A Govett St, Randwick, NSW, 2031, Australia
[c] Department of Pharmacology, Monash University, Wellington Rd, Clayton, Victoria, 3168, Australia

(Received 5 September 1995; accepted 16 October 1995)

Abstract: Three previously reported methanesulfonate insecticides, 6-isobutylthio-2-pyridyl methanesulfonate (**I**) and its sulfoxide (**II**) and sulfone (**III**) analogues were examined in two insect species *Lucilia cuprina* and *Blattella germanica* and in tissues from vertebrates. The results of *B. germanica* tests and cholinesterase assays confirmed the insecticidal activity of the compounds, with cholinesterase inhibition being the most likely mode of insecticidal action. The inactivity of the sulfide **I** and sulfoxide **II** in vertebrate in-vitro studies may indicate that conversion, in vivo, of the sulfide and sulfoxide methanesulfonates to the sulfone (**III**) is a requirement for activity. In mouse toxicity tests, matching high toxicity was observed for the alkylthio-, alkylsulfoxy- and alkylsulfone analogues indicating fast metabolic oxidation of the injected alkylthio- and alkylsulfoxy-compounds. However, in in-vitro tissue tests, the sulfone, although active, did not exhibit the characteristic pharmacological profile of the standard acetylcholinesterase inhibitor, physostigmine. The sulfone demonstrated a mixed action, with indications that it acts as an inhibitor of specific cholinesterase isozymes, or that it may modify responses at cholinoceptors.

Key words: methanesulfonates, sulfoxide, sulfone, cholinesterase inhibition, insecticide, metabolism, vertebrate, cholinergic

1 INTRODUCTION

While the principle of phosphorylation or carbamoylation of the enzyme acetylcholinesterase[1] has been used since World War 2 for the design of organophosphate and carbamate insecticides, mesylation (the reaction with a methanesulfonyl moiety)[2] was used less frequently. The irreversible inactivation of the serine residue of the acetylcholinesterase active site by the first two mechanisms[1] resulted in the development of some hundred commercial insecticides. Some of these were toxic to mammals, e.g. parathion, while others exploited differences between insect and mammalian metabolism to provide insecticides with lower mammalian toxicities, e.g. malathion.

Methane sulfonyl fluoride was recognised some 30 years ago as a potent irreversible mesylating agent of acetylcholinesterase.[3] Hammer *et al.*[4] have used the principle of mesylation and synthesised insecticidal aryl methanesulfonates. More recently Kato *et al.*[5] investigated the insecticidal properties of 6-alkyl-substituted 2-pyridine methanesulfonates, and, in several papers,[5–7] reported an in-vivo activation of 6-alkylthio-2-pyridyl methanesulfonates to the corresponding alkylsulfoxide and alkylsulfone in insects. They claimed that the last oxidation product—the sulfone—was the active inhibitor mesylating acetylcholinesterase. They related the values of inhibition of insect acetylcholinesterase to

* To whom correspondence should be addressed.

Pestic. Sci. 0031-613X/96/$09.00

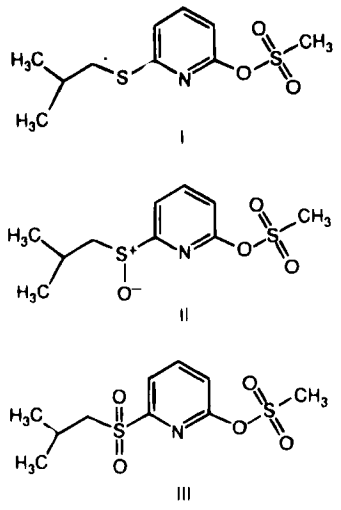

Fig. 1. Structures of methanesulfonates.

insecticidal activity against the rice pest *Nephotettix cincticeps*. Although these patented mesylating agents were reported eight years ago, there are, to date, no commercial insecticides of this structural type. The mammalian cholinesterase inhibitory activities, as well as the pharmacological profiles of the mesylating agents, have also not been reported. To rationalise the synthesis of potential insecticides, the mode of action of three representative insecticidal mesylating agents was examined in vertebrate and insect systems. The compounds examined were: 1-isobutylthio-2-pyridylmethanesulfonate (**I**) and its oxidation products the 6-isobutylsulfoxy- (**II**) and 6-isobutylsulfone (**III**) analogues (Fig. 1).

2 MATERIALS AND METHODS

2.1 Mammalian tissues

2.1.1 Isolated guinea-pig ileum and paced atria

Male Dunkin Hartley guinea-pigs (200–750 g) were killed by cervical dislocation and the heart and ileum were rapidly removed. Segments of ileum (2 cm) were cut and suspended in 30-ml organ baths containing modified Krebs buffer solution of the following composition (mmol litre^{-1}): sodium chloride, 120; potassium chloride, 5; magnesium sulfate, 1; sodium dihydrogen phosphate, 1; sodium hydrogen carbonate, 25; glucose, 11; sucrose, 10 and calcium chloride, 2·5 (added 20 min prior to use). The solution was bubbled with Carbogen (5% carbon dioxide and 95% oxygen) and temperature was maintained at either 32°C or 37°C, pH 7·2. The tissues were placed under 1 g tension and allowed to equilibrate for 1 h with several washings throughout this period. Cumulative concentration–response curves were obtained to acetylcholine in the absence and presence of the test compounds which were incubated for 5–10 min prior to the addition of acetylcholine. Contractions were measured using Grass FTO3 transducers and recorded on a Grass polygraph.

Submaximal responses to Substance P, histamine, 5HT and acetylcholine were obtained and repeated, following an incubation period of 20–30 min, in the presence of compound **III** (7×10^{-7} M).

Antagonists hexamethonium (10^{-4} M), atropine (10^{-7} M), D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}. Substance P ($1{\cdot}8 \times 10^{-6}$ M) and spantide (4×10^{-6} M) were used in an attempt to block an agonist action of compound **III**. High concentrations of 5HT (10^{-5} M) were used to desensitise the tissue and thus antagonise the response to the lower concentration of 5HT. Antagonists were added 30 min prior to the addition of compound **III**.

Following removal of the heart from the guinea-pig, the left and right atrial preparations were dissected and mounted on holders incorporating platinum stimulating electrodes. Each preparation was suspended in a 30-ml bath containing a modified Krebs solution (as above). Tissues were placed under 0·5 g tension and allowed to equilibrate for 1 h. During this time stimulation by means of a Grass S88 stimulator was commenced. Tissues were field stimulated with the following parameters: 0·5 ms, 3 Hz with a voltage of 1–40 V (dial setting). Submaximal doses of acetylcholine ($1{\cdot}7 \times 10^{-7}$ 5×10^{-7} M) and carbachol (5×10^{-8}–1×10^{-7} M) were added by the non-cumulative method. Tissues were incubated for 20 min in the presence of compounds **I** or **III** before exposure to the sub-maximal concentrations of the two agonists.

2.1.2 Toad rectus abdominis

Male Queensland cane toads (*Bufo marinus*) were used. The toads were kept in wet sawdust at 24°C with a photoperiod of 12:12 h light:dark and fed on mealworms. Toads were pithed and the skin covering the abdomen was removed and the rectus abdominis muscle was removed. Four preparations were obtained from each toad and the tissues were suspended in 30-ml baths containing modified Krebs solution (as above). The tissues were placed under 4 g resting tension and allowed to equilibrate for 1 h. Tension was reduced to 1 g prior to the commencement of each experiment. Temperature was maintained at 37°C. Cumulative concentration–response curves for acetylcholine in the absence and presence of compounds **I**, **II** and **III** were obtained. Vehicle controls (5 ml litre^{-1} ethanol in distilled water) were also used.

2.2 Blood assays for anticholinesterase activity

Solutions of compound **I**, **II** or **III** and the ethanol vehicle were tested for any anticholinesterase activity using either guinea-pig, rat or toad blood (heparinised). The solutions were tested on guinea-pig ileum with the exception of the rat blood assay, which was tested on rat ileum. Guinea-pig and rat blood were obtained from

cervical incision prior to removal of tissues. Toad blood was obtained through cardiac puncture of a pithed toad.

The procedure for all blood assays was as follows: Test tubes were set up (as shown in Table 1) to contain 2 ml acetylcholine (ACh; 10^{-5} M) with either 0·2 ml physostigmine (10^{-4} M), 0·2 ml solutions of compounds **I, II** or **III** ($1{\cdot}1$–$1{\cdot}6 \times 10^{-4}$ M), 0·2 ml saline (9 g litre^{-1}) or 0·2 ml ethanol vehicle (5 ml litre^{-1}). Following the addition of approximately 50 µl (volume per drop taken as 10 µl) of blood or saline, the tubes were incubated at 37°C (toad blood was incubated at 30°C) and shaken for 10 min. The reaction was stopped by putting the tubes on ice. Reproducible responses to a sub-maximal concentration of acetylcholine were established on guinea-pig or rat ileum prior to the addition of the test aliquots. Knowing the sensitivity of the assay tissue, an appropriate volume from each tube was added to the tissue to give a submaximal contraction. Concentrations stated are approximate only due to the inaccuracy inherent in measuring the volumes of saline and blood added dropwise.

2.3 Assay of bovine, fly head and *Electrophorus electricus* acetylcholinesterase and human butyrylcholinesterase inhibition

Using a combination of the methods of Ellman *et al.*,[8] Devonshire and Sawicki[9] and Devonshire and Moores,[10] assays were set up to test these compounds for their anti-cholinesterase activity. Fly head cholinesterase was extracted from the blowfly (*Lucilia cuprina* as described by Devonshire and Moores[10] and bovine, human and *Electrophorus* cholinesterases were purchased from Böhringer Manheim. Test compounds were added to a buffered solution with the substrates acetylthiocholine iodide or butyrylthiocholine iodide with a known quantity of the enzyme, and incubated at 37°C for 10 min using a BIO-RAD Enzyme Analyser. At the end of 10 min the reaction was stopped by the addition of 1 g litre^{-1} physostigmine sulfate (eserine) solution.

TABLE 1
Procedure for Blood Assays

Tube	*2 ml* (10^{-5} *M*)	*Five drops* (≈*10 µl*) *per drop*)	*0·2 ml*
A	ACh	Blood	Saline
B	ACh	Saline	Saline
C	ACh	Blood	Physostigmine
D	ACh	Saline	Physostigmine
E	ACh	Blood	Compounds **I/II/III**
F	ACh	Saline	Compounds **I/II/III**
G	ACh	Blood	Ethanol vehicle
H	ACh	Saline	Ethanol vehicle

The absorbence (405 nm) readings at 10 min were used for IC_{50} calculations. The IC_{50} values shown are calculated from a minimum of eight replicates.

2.4 Protocol for topical application of compounds to insects

Adult (five to nine days old) male blowflies (*L. cuprina*) from laboratory culture were lightly anaesthetised with ether and dosed with 1 µl of serial dilutions of test solution (4 mg ml^{-1} compound in acetone) using a Burkard Micro-applicator. After treatment, the flies were supplied with sugar and water and left for 24 h at 26°C. At that time mortalities were counted and recorded. Four replicates consisting of 10–15 flies per dose were used, where the average weight was 2·5 g per 100 flies.

Adult (six to ten weeks old) male German cockroaches (*Blattella germanica*) from laboratory culture were immobilised with carbon dioxide, and dilutions of test compounds were applied in the same way as for blowfly testing except that the solution was placed at the base of the coxae in order to ensure even dosing. Four replicates consisting of 10–15 cockroaches per dose were used, where the average weight was 5 g per 100 cockroaches.

2.5 Protocol for mouse toxicity tests

Male BALB/C mice (20–25 g) were used for toxicity testing. Compounds were dissolved in olive oil (which itself had no effect) and injected i.p. at 100, 10 and 1·0 mg kg^{-1}. Five to ten mice were dosed at each concentration, and five control mice were dosed with vehicle.

The mice were observed at half-hourly intervals and symptoms recorded. Further readings were taken for the next seven days after which the surviving mice were killed by ether inhalation.

2.6 Drugs

I, II and **III** were synthesised at the Division of Chemicals & Polymers, CSIRO. Other drugs used, acetylcholine chloride, carbachol chloride, physostigmine sulfate (eserine), atropine sulfate, nicotine sulfate, histamine dihydrochloride, 5-HT sulfate (serotonin) and Substance P, were purchased from Sigma. Spantide and D-Arg^{1}, D-Pro^{2}, D-Trp^{7,9}, Leu^{11}. Substance P were purchased from Auspep and hexamethonium iodide from Koch.

2.7 Statistical analysis

Analysis for statistical significance of results was performed using the standard formulae for calculating mean ± standard error of the mean (SEM) and using

Student's paired t-test and Student's unpaired t-test as appropriate. Significance was taken as being $P < 0.05$. Percentage inhibition was calculated where appropriate. LD_{50} and IC_{50} values were obtained by probit analysis.

2.8 Ethics

Ethical approval was obtained from the Standing Committee in Ethics in Animal Experimentation, Monash University, and the University of NSW for all animal testing used in this study.

3 RESULTS

3.1 Effects of compounds I, II and III determined using blood cholinesterase bioassays

Compounds **I**, **II** and **III** failed to show inhibition of guinea-pig blood cholinesterase ($n = 3$ replicates for each compound, using different animals; when tested on guinea-pig ileum). Compound **II** did not inhibit rat blood cholinesterase when tested on rat ileum ($n = 1$). Compound **III** did not inhibit toad blood cholinesterase when tested on the more sensitive guinea-pig ileum ($n = 1$). Physostigmine (approx. 10^{-5} M) was used as a control with each assay, and effectively inhibited the cholinesterase activity from the blood of each species tested ($n = 11$). The ethanol vehicle was without effect ($n = 11$).

3.2 Effects of compounds I, II and III on the response to acetylcholine of toad rectus abdominis

Compounds **I**, **II** and the ethanol vehicle (0·056 ml litre^{-1}, $n = 3$) were without effect on responses to acetylcholine in this preparation. Data for compound I and vehicle are shown in Fig. 2.

Compound **III** ($n = 3$) and physostigmine ($n = 2$) were both found to potentiate cumulative concentration–response curves to acetylcholine. Using Student's unpaired t-tests, compound **III** was found to have a significant effect ($P < 0.05$) at a concentration of 2.15×10^{-6} M (see Fig. 2).

3.3 Effects of compounds I, II and III on the response of guinea-pig ileum to agonists

The ethanol vehicle (0·05 ml litre^{-1}, $n = 4$) did not have any effect on cumulative concentration–response curves to acetylcholine in the isolated guinea-pig ileum. Pilot experiments using compounds **I** (1.5×10^{-6} M) and **II** (1.64×10^{-6} M) showed that, at these concentrations, the compounds had no effect on the concentration–response curves to acetylcholine or on the basal tone of the ileum. In contrast, compound **III** (4.1×10^{-6} M) induced phasic contractile responses in the isolated guinea-pig ileum ($n = 12$). Atropine (10^{-7} M, $n = 3$) attenuated but did not completely abolish these contractions, which were not affected by hexamethonium (10^{-4} M). Test responses to submaximal concentrations of the agonists acetylcholine and histamine were effectively blocked by the antagonist atropine at a concentration of 10^{-5} M, and responses to submaximal concentrations of 5-HT were attenuated by prior exposure to a high concentration of 5-HT (10^{-5} M). Compound **III** was tested in the presence of 5-HT (10^{-5} M, $n = 3$) or spantide (4×10^{-6} M, $n = 2$), and it was found that these two antagonists did not affect the response to compound **III**. The Substance P antagonist D-Arg^1, D-Pro^2, D-Trp^{7,9}, Leu^{11}, Substance P (1.8×10^{-6} M,

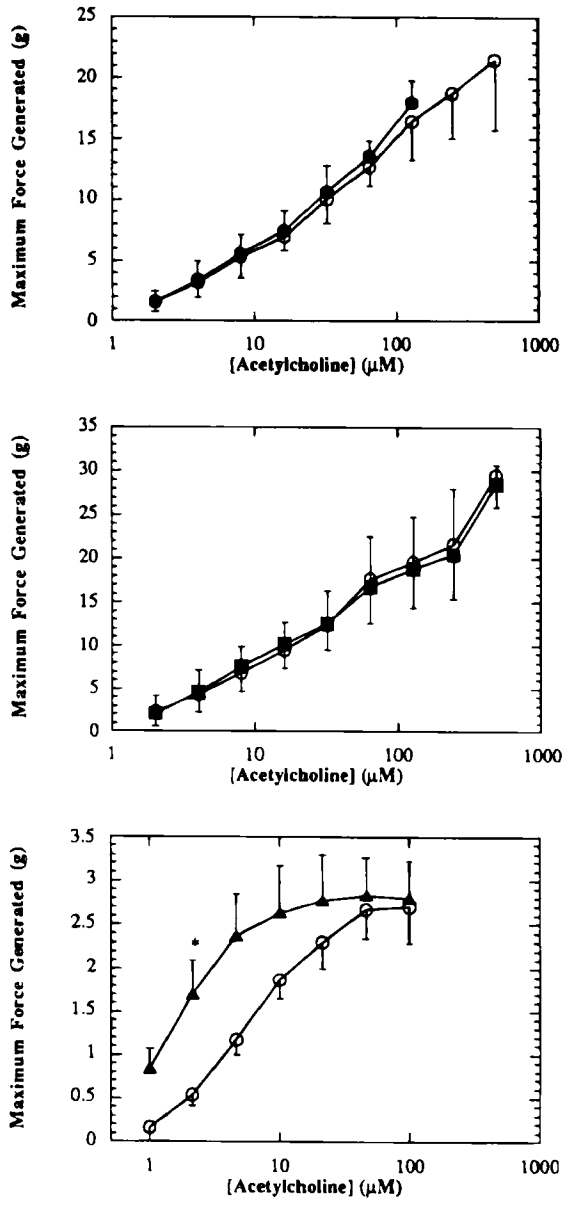

Fig. 2. Concentration–response curves obtained to acetylcholine (○) alone, and in the presence of: (●) ethanol (0·056 ml litre^{-1}); (■) Compound **I** (1·4–1·7 μM) and (▲) Compound **III** (4·1 μM) in toad rectus abdominis. * Indicates significance in Student's unpaired t-test ($P < 0.05$).

TABLE 2
Cholinesterase Inhibition, IC_{50} (μM)

Compound	*Fly head (L. cuprina) acetylcholinesterase*	*Bovine erythrocyte acetylcholinesterase*	*Human erythrocyte butyrylcholinesterase*	*Electrophorus electricus acetylcholinesterase*
I	N/A[a]	N/A	N/A	884
II	633	633	N/A	42
III	416	551	N/A	84
Physostigmine	0·03	14·49	15·19	0·62

[a] N/A indicates minimal or no effect on cholinesterase activity when tested at 1 mM.

$n = 1$) was also tested but did not affect the contractions induced by compound **III**. However, submaximal concentrations of Substance P were not blocked by spantide nor by D-Arg[1], D-Pro[2], D-Trp[7,9], Leu[11], Substance P, therefore these two agents do not appear to be effective antagonists of Substance P on the guinea-pig ileum at the concentrations used.

Low concentrations of compound **III** (7×10^{-7} M) did not induce contractions of the ileum *per se* and did not affect submaximal responses to acetylcholine ($n = 3$), histamine ($n = 2$), 5-HT ($n = 3$) or Substance P ($n = 3$). Although responses to acetylcholine, histamine and 5-HT were not significantly affected by compound **III**, responses to Substance P were greater in the presence of compound **III**, although this effect did not attain statistical significance when analysed using Student's paired *t*-test.

3.4 Effects of compounds I and III on responses of guinea-pig atria to agonists

In the paced guinea-pig atria, submaximal concentrations of acetylcholine reduced electrically evoked contractions due to action on inhibitory muscarinic receptors. Compound **I** ($4{\cdot}4 \times 10^{-6}$ M, $n = 4$) and the ethanol vehicle (0·15 ml litre^{-1}, $n = 6$) had no effect on this preparation. However, the presence of compound **III** (4×10^{-6} M, $n = 4$) was found to increase the inhibition caused by acetylcholine ($P < 0{\cdot}05$, paired Student's *t*-test). Compound **III**, however, had no effect on the magnitude of contractions when added alone (4×10^{-6} M), or on the response to submaximal concentrations of carbachol in this preparation. Physostigmine (10^{-6} M, $n = 3$) reduced the height of the contractions between 12 and 70% when added alone (data not shown).

TABLE 3
Toxicity in Mice

Compound	*Mortalities*		
	1 mg kg^{-1}	*10 mg kg^{-1}*	*100 mg kg^{-1}*
I	0/5	10/10	10/10
II	0/10	7/9	10/10
III	0/5	4/5	5/5

3.5 Cholinesterase inhibition

The results of in-vitro assays to assess the degree of cholinesterase inhibition using cholinesterases from various sources by compounds **I**, **II** and **II** are summarised in Table 2. Physostigmine was an effective inhibitor of acetylcholinesterase from all sources, whereas compounds **I**, **II** and **III** showed differing patterns of cholinesterase inhibition (Table 2).

3.6 Toxicity in mice and insects

Results of toxicity testing in mice and insects are shown in Tables 3 and 4 respectively.

4 DISCUSSION

Our tests on *B. germanica* (German cockroach) confirmed the insecticidal activity of the parent compound

TABLE 4
Toxicity in Insects

Compound	*Lucilia cuprina* LD_{50} (*mg kg^{-1}*)	*95% confidence limit*		*Blattella germanica* LD_{50} (*mg kg^{-1}*)	*95% confidence limit*	
		Lower	*Upper*		*Lower*	*Upper*
I	12·98	10·82	15·58	1·82	1·67	2·36
II	58·09	24·29	138	0·73	0·58	0·88
III	>100			1·4	0·98	1·78

6-isobutylthio-2-pyridyl methanesulfonate (**I**), its sulfoxide (**II**) and sulfone (**III**). These results agree with the insecticidal activity found by Kato *et al.* on strains of *N. cincticeps*.[6] This activity most probably results from inhibition of cholinesterase, as indicated in biochemical assays of fly head acetylcholinesterase. However, the sulfone (**III**) was found to be inactive when applied to the blowfly, *L. cuprina*, which may be due to its lower lipid solubility and consequent lack of penetration through the insect cuticle.

Bioassays of cholinesterase activity indicated that none of the compounds inhibited the cholinesterase present in the blood of the vertebrate species tested, whereas physostigmine effectively inhibited all the cholinesterases tested. One explanation of this may be that the maximum concentration of solutions achievable with these compounds ($4{\cdot}1 \times 10^{-4}$ M for **III**) may have been too low to inhibit effectively the cholinesterases present. Alternatively, Chatonnet and Lockridge[11] have reported the presence of at least two forms of cholinesterase in whole blood; the membrane-bound acetylcholinesterase, and butyrylcholinesterase which is found in serum. They also reported that acetylcholinesterase can exist in several different forms, many of which are found in mammals. Our in-vitro assay showed that none of the compounds inhibited human butyrylcholinesterase, and it is most likely that these compounds do not equally inhibit all forms of cholinesterase found in other species.

The sulfone, like physostigmine, enhanced acetylcholine-induced contractions of the toad rectus abdominis. This potentiation would be expected if sulfone was acting at the nicotinic receptors present or was inhibiting cholinesterase at the motor endplate. A test of inhibition of toad blood cholinesterase showed the sulfone to be without effect.

In guinea-pig ileum, which contains a heterogeneous population of receptors for acetylcholine, including muscarinic and nicotinic receptors,[12,13] the sulfone, at a concentration of 4 μM, induced phasic contractions which were attenuated by the muscarinic antagonist atropine but not by the nicotinic antagonist hexamethonium. At lower concentrations, the sulfone potentiated submaximal responses to Substance P (SP) which could be a result of inhibition of the peptidases which metabolise Substance P. Chatonnet and Lockridge,[11] reported that both acetylcholinesterase and butyrylcholinesterase are among the enzymes that can hydrolyse Substance P. Thus, the potentiation of Substance P in the guinea-pig ileum by compound **III** could be the result of inhibition of an isozyme of cholinesterase or other SP-degrading enzyme.

In paced atria from guinea-pig, the sulfone enhanced the negative inotropic effect of acetylcholine, which is mediated by muscarinic receptors. The sulfone was without effect on the negative inotropic response to carbachol (which, unlike acetylcholine, is not subject to rapid metabolism by cholinesterases). The sulfone was, however, without effect when added alone. This is in contrast to physostigmine, which reduces twitch height in electrically stimulated atria due to its inhibition of cholinesterase.

The lack of effect of the parent alkylthio compound (**I**) and its corresponding sulfoxide (**II**) in all the in-vitro tests using mammalian and amphibian tissue may indicate that conversion to the higher oxidation state (**III**) is required for activity. The mouse toxicity experiments gave approximately the same high toxicity levels for all three compounds, which indicates rapid metabolic conversion of **I** and **II** to the sulfone (**III**) in the intact animal. However, in contrast to the proposed mode of action in insects, the sulfone did not give the profile of responses expected of a standard cholinesterase inhibitor.

We conclude that, in vertebrates, the sulfone alone may inhibit an isozyme/s of cholinesterase in certain tissues. In addition, it may selectively act at a subtype of muscarinic/nicotinic receptor.

ACKNOWLEDGEMENTS

Thanks are due to G. Wernert and R. Walser of CSIRO, Division of Chemicals & Polymers, for synthesis of compounds **I**, **II** and **III**.

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